Immunization with a peptide mimicking Lipoteichoic acid protects mice against *Staphylococcus aureus* infection

Xia-Yu Yi [a,1], Zhao-Xia Huang [a,1,2], Xiao-Rui Hou [a], Ping Zhu [a], Xiang-Yu Wang [a,3], Hai-Bo Luo [b], Bei-Yi Liu [a,*]

[a] *Department of Immunology, School of Basic Medical Sciences, Southern Medical University, Guangzhou, Guangdong, China*
[b] *Clinical Laboratory, The Seventy-fourth Military Hospital of the PLA, Guangzhou, Guangdong, China*

ABSTRACT

Lipoteichoic acid (LTA), a major component of the cell wall of *Staphylococcus aureus* (*S. aureus*), is not generally considered as an ideal vaccine candidate since it is a thymus-independent antigen. In this study, we screened a 12-mer phage peptide library and identified a series of peptide sequences that can mimic the epitope of LTA. A tetra-branched multiple antigenic peptide, named MAP2-3, comprising one of the positive peptide sequences (GHKEDRQWCQHS), was synthesized. Immunization with MAP2-3 induced LTA-specific IgG antibodies, prolonged the survival time, and decreased the bacterial burden in organs of mice infected with *S. aureus*. Moreover, passive immunization with polyclonal anti-MAP2-3 sera reduced bacterial load in organs of mice with bacteremia, alleviated acute lung injury in mice with pneumonia, and decreased the size of lesions in mice with skin infection. The number of LTA-specific antibody-secreting cells in the spleen of MAP2-3 immunized mice were significantly higher than that in the control mice. In summary, as a surrogate of LTA, vaccination with MAP2-3 elicited humoral immune response and protected mice from *S. aureus* infection. This study provides a new option to design vaccines against *S. aureus*.

© 2019 Elsevier Ltd. All rights reserved.

1. Introduction

S. aureus, one of the most common Gram-positive bacteria colonizing human hosts, can cause a broad spectrum of diseases including skin and soft tissue infections, bacteremia, pneumonia, and septic shock [1,2]. The emergence of some antibiotics-resistant strains, such as methicillin resistant *S. aureus* (MRSA), makes the treatment tougher and leads to higher healthcare costs and mortality rate [3,4]. Given this fact, there is a need to develop novel immunoprophylaxis and immunotherapy strategies against *S. aureus*.

Lipoteichoic acid (LTA) is an essential component of cell wall that is expressed steadily on the surface of *S. aureus*. The structure of LTA varies and has been grouped into five different types (type I

to V). Type I LTA, found in a large range of Gram-positive bacteria such as *S. aureus*, *Bacillus subtilis* (*B. subtilis*) and *Listeria monocytogenes*, has been well characterized [5]. The basic structure of type I LTA backbone is formed by conserved, unbranched, repeating units of 1, 3-glycerolphosphate that is linked to the cytoplasmic membrane via a glycolipid anchor. The hydroxyl groups of the C2 position of glycerol are modified with D-alanylation and/or glycosylation [5,6]. Recent works have demonstrated that LTA is essential for the pathogenicity and survival of *S. aureus* under low-osmolarity conditions [5]. D-Alanylation of LTA regulates autolysis [7], host cell adhesion [8], biofilm formation [9], as well as penetration of blood-brain barrier [10]. Moreover, LTA and another cell wall component peptidoglycan (PGN), can act in synergy to induce systemic inflammation and multiple organ failure [11]. Despite the fact that LTA is a crucial component of *S. aureus*, as a thymus-independent antigen (TI antigen), it has weak immunogenicity and cannot sufficiently induce immune memory [5].

Phage-displayed peptide library is a useful tool for identifying peptide sequences that mimic epitopes of carbohydrates expressed on tumor cells or bacteria cell surface [12]. Peptide mimic coupled with carrier proteins, acting as a thymus-dependent antigen (TD antigen) and a surrogate for carbohydrate, has been used as a vaccine candidate to induce immune responses, thereby protecting the hosts from infections [13–16]. However, peptide conjugated

carrier proteins might be associated with some undesirable effects including the induction of antibodies against the protein itself and suppressing the production of anti-peptide antibodies [17]. As an alternative, multiple antigenic peptide (MAP) has been used as an immunogen for developing vaccines against various pathogens [18–21]. MAPs are synthetic peptides in which lysine residues are used as the scaffolding core to support the formation of maximally eight branches with varying or the same peptide sequences. Compared to linear peptide, MAP is more stable and big enough for immunization without crosslinking with any carrier protein [18].

In this study, we screened a phage displayed peptide library and obtained a series of peptide that can mimic the epitope of LTA. A MAP derived from a positive sequence was synthesized. We further investigated the antigenicity, immunogenicity, and protective properties of this MAP in mouse models infected with S. aureus.

2. Materials and methods

2.1. Phage peptide library, LTA, and antibodies

A PhD-12 phage display peptide library kit (Cat No. E8110S) was purchased from New England Bio Lab Inc. LTA derived from S. aureus (Cat No. L2515) was purchased from Sigma. Monoclonal antibody against LTA from many Gram-positive bacteria (Cat No. MA1-7401) was purchased from ThermoFisher Scientific. Another LTA mouse mAb (Cat No. LS-C102921), reacting with LTA from Listeria monocytogenes, was purchased from LifeSpan Biosciences Inc. Details about other antibodies and reagents used in this study are summarized in Supplementary Tables 1 and 2.

2.2. Preparation of bacteria

S. aureus (methicillin sensitive S. aureus, MSSA, ATCC 25923), S. epidermidis (ATCC 12228), S. pyogenes (ATCC 19615), E. coli (ATCC 25922), E. faecium (ATCC 35667), and E. faecalis (ATCC 29212) were purchased from Wenzhou Kont Biology and Technology. All the bacteria were grown in tryptic soy broth (TSB) or LB at 37 °C with 250 rpm shaking for overnight. Cells were then collected, washed, and diluted with sterile phosphate buffered saline (PBS) to an appropriate concentration.

2.3. Animals and ethics statement

BALB/c mice and C57BL/6 mice (5–6 weeks old, female), and New Zealand rabbit (female, average weight: 1 kg) were purchased from the Experimental Animal Center, Southern Medical University, Guangzhou, China. All of the animal experiments were approved by the Institutional Animal Care and Use Committee of the Southern Medical University (permit number: L2015070) and carried out in strict accordance with national guidelines for animal welfare. S. aureus was not detected by ELISA in serum samples of all animals before immunization.

2.4. Panning clones from phage peptide library

Clones that mimic LTA were screened from the phage peptide library by using both surface panning and solution-phase panning procedures. In surface panning procedure, phage particles (1.0×10^{11} pfu) were added to the plate coated with 20 µg/ml anti-LTA mAb (MA1-7401) and incubated at room temperature for 60 min with gentle rotation. Bound particles were eluted with 50 µl 0.2 M glycine/HCl buffer (pH 2.2) containing 1 mg/ml BSA, followed by neutralization with 1 M Tris/HCl buffer (pH 9.1). Solution-phase panning with affinity bead capture was also performed. Briefly, phage particles (1.0×10^9 pfu) was incubated with

10 ng mAb MA1-7401 for 20 min at room temperature, mixed gently with the Protein A/G-agarose that had been blocked with 5 mg/ml BSA, and incubated for 15 min. The beads were then washed, and the bound phage clones were eluted as described for the surface panning procedure. For both panning procedures, the eluted phages were titrated, amplified and purified for the next round of screening. After three rounds of panning, plaques randomly selected from the titering plates were analyzed by ELISA.

2.5. Detection of phage clones by ELISA and sequencing of phage clones

96-well microplates were coated with MA1-7401 mAb or PBS as a control at 4 °C for overnight followed by blocking with 1% casein. The phage-containing supernatant from each individual clone was added into the wells and incubated at 37 °C for 30 min. HRP-conjugated anti-M13 mAb was then added (37 °C for 30 min). Color reactions were developed by using tetramethylbenzidine (TMB) with hydrogen peroxide. Plates were read at 450 nm using an ELISA reader (Perkin Elmer precisely 2030 Multilabel Reader, Victor™ X4).

Positive clones identified by ELISA were amplified, and their single-stranded DNA was isolated for DNA sequencing by Invitrogen Inc. (Shanghai, China).

2.6. Synthesis of linear peptides and MAPs that mimic LTA

All peptides, including the linear peptides and tetra-branched multiple antigenic peptides were synthesized with purity above 90% by Hybio Pharmaceutical (Shenzhen, China). Peptides L3 and MAP3 were dissolved in DMSO at 100 mg/ml, and other peptides were dissolved in endotoxin-free water (Sigma) at 50 mg/ml. All peptides were diluted in sterile PBS when used in assay.

2.7. Detection of synthetic peptides by ELISA

For examination of linear peptides, Biotin-labeled peptides were added into the wells coated with 5 µg/ml mAb against LTA (MA1-7401 or LS-C102921) and incubated at 37 °C for 30 min, followed by detection with HRP-labeled streptavidin. For examination of MAPs, 5 µg/ml mAb against LTA was added to wells coated with different MAPs and incubated at 37 °C for 40 min. HRP-labeled goat anti-mouse IgG was then added followed by color reaction for detection.

2.8. Immunization with MAPs and detection of immunized sera

The protocol of vaccination was based on our previous study with slight modifications [21]. Briefly, BALB/c or C57BL/6 mice (5–6 weeks old, female) were randomly divided into three groups. The first two groups were subcutaneously (s.c.) injected with MAP2-3 and MAPctrl (a lysine-based, tetra-branched scaffold used as control MAP) (100 µg/mice), respectively, for four to five times at 2-week intervals. The first immunization was performed in Freund's complete adjuvant (Cat No. F5881, Sigma) and the subsequent booster immunizations were administered in Freund's incomplete adjuvant (Cat No. F5506, Sigma). The third group of mice, without any peptide immunization, was breed at the same time and used as a blank control. Serum samples were collected through retro-orbital bleeding 7 days after each immunization cycle and frozen at −70 °C for later use.

The specificity of antibody in serum samples was measured by indirect ELISA. Briefly, MAP2-3 (10 µg/ml), LTA, PGN, and LPS (each in concentration 10 µg/ml), as well as ultrasonic products of bacteria (S. aureus, S. edpidermidis, S. poygens, E. faecium, and E. coli) were coated in 96-well plates at 4 °C for overnight. All wells except those coated with S. aureus ultrasonic products were blocked with 0.25% casein. To avoid binding of staphylococcus protein A to IgG, wells

coated with *S. aureus* ultrasonic products were blocked with 0.25% casein containing 1% guinea pig sera. Serum samples were subsequently added as primary antibodies, followed by incubation with HRP-conjugated goat anti-mouse IgG (H + L). The titer of antibody was defined as the highest dilution that gives more than two times the absorbance values of the blank control.

To determine the class/subclass of immunoglobulin (Ig) in serum samples, the sera (1:100 dilution) were added into the wells coated with LTA from *S. aureus* and incubated at 37 °C for 40 min. Plates were then washed followed by incubation with isotype specific reagents (including goat anti-mouse IgG1, goat anti-mouse IgG2a, goat anti-mouse IgG2b, goat anti-mouse IgG3, goat anti-mouse IgM, and goat anti-mouse IgA) at 37 °C for 30 min. The wells were washed and incubated with HRP-labeled rabbit anti-goat IgG at 37 °C for 30 min.

To prepare rabbit anti-MAP2-3 sera, New Zealand rabbits were immunized by intradermal injection with 1 mg MAP2-3 emulsified with Freund's adjuvant at day 0, 21, 35 and 49. The specificity and titer of rabbit serum samples were determined by ELISA as described for mice samples. Seven days after the last immunization, the rabbits were anesthetized with 20% urethane (5 mg/kg), and serum samples were collected for opsonophagocytosis assay and passive immunization.

2.9. Opsonophagocytosis killing assay

Modified opsonophagocytosis killing assay was performed as previously described [22,23]. Briefly, mouse neutrophil granulocytes were isolated by using anti-Ly-6G microbead kit with purity up to 90% as determined by flow cytometry. *S. aureus* (1×10^3 CFU) was first incubated with heat-inactivated rabbit antisera (final dilution was 1:400) at room temperature for 10 min. Purified mouse neutrophil cells (1×10^5 cells/well) together with fresh guinea pig serum (final concentration: 1%) used as complement were then added to the bacteria/antisera mixtures. Plate was then under agitation at 150 rpm at 37 °C for 1 h. Samples were cooled on ice for 20 min to terminate the reactions and transferred to TSA plates (10 μl/well) for calculating colony forming unit (CFU). Percent killing (%) = [1-CFUs in wells (sera + complement + cells + bacteria)/CFUs in wells (cells + bacteria + complement)] × 100%.

2.10. Systemic infection in mouse with active immunization

Experiments using systemic infection models were performed as previously described [21]. Briefly, BALB/c or C57BL/6 mice were infected with *S. aureus* (1×10^8 CFU/mouse in septic shock model or 2×10^7 CFU/mouse in bacteremia model) via tail vein injection (*i.v.*) 7 days after the last immunization. The mortality was monitored for 10 days in the septic shock model and the bacterial load in tissues was measured at day 3 in the bacteremia model.

2.11. Mouse models for passive immunization

Three different mouse models were created by using BALB/c mice (female, 6–7 weeks old) for passive protection experiments as described in previous studies with minor modifications [22,24]: (1) Bacteremia model: rabbit sera (100 μl/mouse, *i.p.*) were given to the mice 24 h prior to *S. aureus* challenge (2×10^7 CFU/mouse, *i.v.*). The bacterial load in blood and organs were determined at indicated time points. (2) Pneumonia model: Bacteria (4×10^9 CFU/ml, 10 μl) and serum samples (10 μl) were mixed and intranasally administered to mice anesthetized with pentobarbital sodium (50 mg/kg, i.p.). After infection for 24 h, the lung tissue was aseptically isolated and the bacterial burden was determined. (3) Skin abscess and dermonecrosis model: *S. aureus* (2×10^7 CFU/mouse), together with the antisera, was subcutaneously injected in the shaved flank of anesthetized mice. The skin abscess mass and dermonecrotic area was monitored for 14 days. Area of dermonecrotic lesion was measured by using a standard formula [$Area = (\pi/2) \times length \times width$] according to Bagnoli F et al.'s work [22].

2.12. Measurement of bacterial burden and histopathology

BALB/c or C57BL/6 mice were sacrificed at indicated time points after infection. Blood was aseptically collected. The spleen, unilateral kidney, lungs or skin abscess were aseptically isolated and
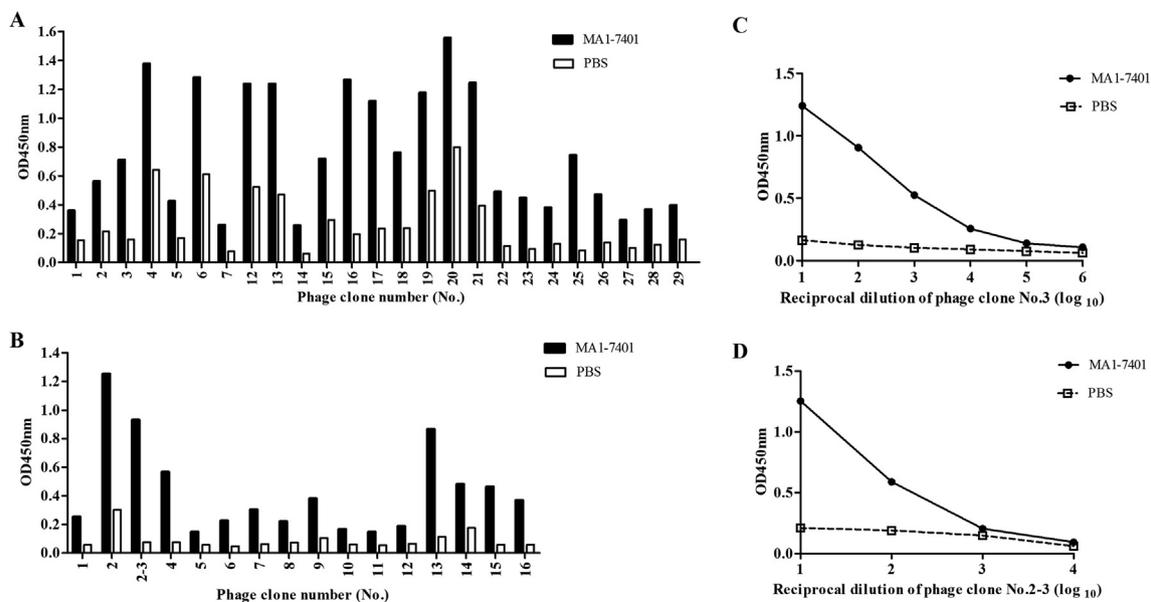

Fig. 1. Reactivity of phage clones with anti-LTA mAb (MA1-7401) detected by ELISA. (A) Binding assay for phage clones identified from surface panning. (B) Binding assay for phage clones from solution-phase panning. (C) Dose-dependent binding assay for phage clone No. 3 from surface panning. (D) Dose-dependent binding assay for phage clone No. 2–3 from solution-phase panning.

Table 1
Peptides identified from phage display library screen.

Phage clones identified	Amino acid sequences
Surface panning procedure	
3,5,15,22,23,26,29	VH**WDF**RQ**W**WQP**S**
4,13,16,19	WHWSWIYSTMNP
6	IHWWWAGSRTT
12	WHLWPSWWRNDT
18	LTFYPVRTQSTH
20	WHWDKWWYYQVE
28	FHWWLSTQPYGT
25	WNIEYVMGYMTL
Solution phase panning procedure	
2	G**HWDF**RQ**W**WQP**S**
2–3	G**HKEDRQWCQHS**
4	APVAPFLSGWQL
13	MYNEPARPPVGL
14	VH**WDF**RQ**W**WQP**S**
15	RKVKRRPRVSNL
16	GISSFPIRAASP

Conserved residues in peptides were highlighted in black.

homogenized with 2 ml sterile PBS or PBS containing 0.01% Triton X-100 (for skin). Blood and tissue homogenates were then plated on agar media and CFUs were determined after overnight culture at 37 °C. For histological analysis, tissue sections were stained with hematoxylin and eosin (H&E) and images were obtained using a Nikon eclipse Ni light microscope (Japan) [25].

2.13. Splenocytes isolation

One month after the last immunization, BALB/c mice were injected intraperioneally with heat-killed *S. aureus* (2×10^7 CFU/mouse). Five days later, splenocytes were prepared by squeezing the spleen in RPMI 1640 medium. Erythrocytes were lysed with RBC lysis buffer (eBioscience). After being washed with RPMI1640, splencoytes were resuspended with RPMI1640 medium containing 10% calf serum.

2.14. ELISPOT assay

Enzyme-linked immunospot (ELISPOT) plates (MultiScreen-IP Filter Plate, MAIPS4510, Millipore) were coated with LTA (10 µg/ml) or heat-inactivated *S. aureus* (2×10^4 CFU/well) and incubated at 4 °C for overnight. The plates were blocked with RPMI1640 medium containing 10% calf serum at room temperature for 2 h. Splenocytes (4×10^5/well) were then added into the wells and incubated at 37 °C for 24 h. Cells and the medium were decanted from the plates, and the plates were washed for three times with 0.05% PBST. HRP conjugated goat anti-mouse IgG (H + L) was added subsequently and incubated at 4 °C for overnight. The plates were extensively washed and developed with substrate solution containing AEC (Sigma) and H_2O_2. The reaction was terminated with H_2O. After drying, the plates were read using a CTL analyzer (CTL S5 micro, Cellular Technology Ltd.).

2.15. Statistical methods

Numeric measurements were expressed as means ± SEM. Five to eighteen mice were used in each experiment, and experiments under the same condition were repeated two to three times. For peptide antigenicity and antibody specificity, the comparison was performed using either one-way ANOVA or Student's *t*-test. For comparison of ELISPOT data, nonparametric test was used. For
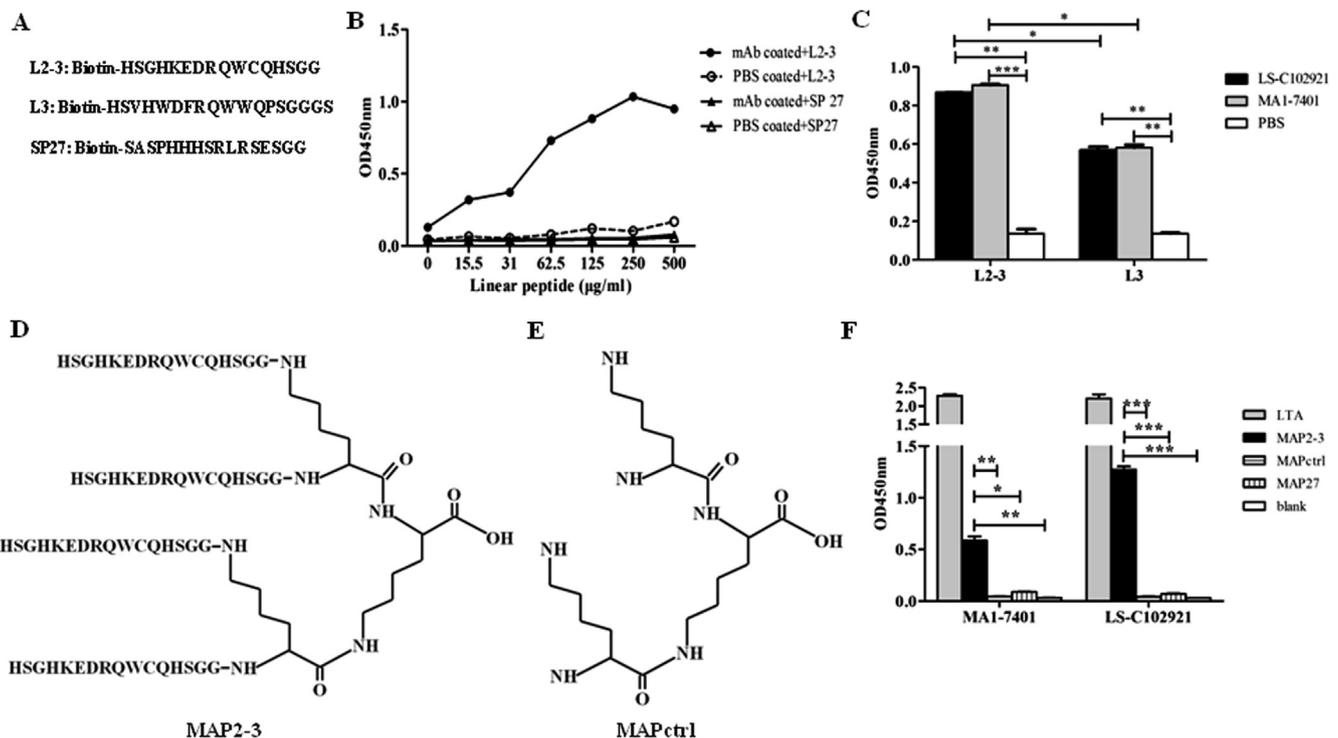

Fig. 2. Reactivity of synthetic peptides with LTA-specific mAbs determined by ELISA. (A) Sequences of the three linear peptides: L2-3 sequence was derived from clone 2–3 (GHKEDRQWCQHS) from solution-phase panning, L3 sequence was derived from clone 3 (VHWDFRQWWQPS) from surface panning, SP27 is a linear peptide mimicking PGN epitope. (B) Binding of L2-3 in a serial dilution with MA1-7401 mAb, a monoclonal antibody specifically reacts with LTA from different Gram positive bacteria. (C) Binding of L2-3 or L3 linear peptide with MA1-7401, and LS-C102901, another antibody specifically reacts with LTA from *Listeria monocytogenes*. (D) Structure diagram of MAP2-3 (molecular weight: 7726.3). (E) Structure diagram of MAPctrl, a lysine-based tetra-branched scaffold used as control MAP (Molecular weight: 402.5). (F) Binding of MAP2-3 with two different LTA-specific mAbs. Data are expressed as mean ± SEM. For statistical analysis, least significant difference (LSD) in one-way ANOVA or Student's *t*-test was performed to examine the difference in antibody reactivity between the two peptides. *p*-values: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

comparison of bacterial load between groups, Mann-Whitney U of non-parametric test was used. For comparison of survival rate in murine lethal challenge model, log-rank test was used. P-value < 0.05 was considered to be statistically significant. All statistical analyses were performed using SPSS version 20.0 software.

3. Results

3.1. Identification of phage clones

We screened a phage displayed peptide library to identify peptides that can mimic the epitope of LTA. After three rounds of screening, phage clones were isolated and the binding specificity was examined by ELISA. Seventeen out of the 29 phage clones from the surface panning procedure (Fig. 1A), and 7 out of the 16 clones from the solution-phase panning (Fig. 1B) specifically bound to MA1-7401 mAb, an antibody recognizing LTA from many Gram positive bacteria. The peptide sequences displayed on the positive clones are summarized in Table 1. A same sequence VHWDFRQWWQPS was expressed on eight different clones, of which 7 were from the surface panning (No. 3, 5, 15, 22, 23, 26, 29), and 1 was from the solution-phase panning (clone 14). Besides, sequence expressed on clone 2 (GHWDFRQWWQPS) from the solution-phase panning is nearly identical to the above sequence, except that the first amino acid is glycine instead of valine. Moreover, the sequence displayed on clone No. 2–3 (GHKEDRQWCQHS) from the solution-phase panning is also similar to the above two sequences, as they all have some conserved amino acids as shown in Table 1. ELISA experiments further confirmed that sequence VHWDFRQWWQPS (expressed in clone 3 from the surface panning) and sequence GHKEDRQWCQHS (clone 2–3 from the solution-phase panning) reacted with LTA-specific mAb in a dose-dependent manner (Fig. 1C–1D).

3.2. Synthetic peptides specifically bound to anti-LTA Abs

Two biotin-labeled linear peptides, L2-3 (containing the core sequence GHKEDRQWCQHS) and L3 (containing the core sequence VHWDFRQWWQPS) were synthesized. To maintain the peptide structure initially displayed on phage clones, the two core sequences were flanked by amino acids HS at N-terminus and GG/GGGS at C-terminus (Fig. 2A). ELISA results showed that both L2-3 and L3 peptides could react with LTA-specific mAb (MA1-7401) in a dose-dependent manner (Fig. 2B and Suppl. Fig. 1A). In contrast, SP27, a peptide that has been proved to mimic the epitope of peptidoglycan (PGN) [21], could not bind to the anti-LTA mAb (Fig. 2B). Moreover, both L2-3 and L3 could bind to LS-C102921 mAb, another anti-LTA mAb which recognizes LTA from *Listeria monocytogenes* (Fig. 2C). The reactivity of L2-3 to MA1-7401 or LS-C102921 mAb was significantly stronger than that of L3 at the same concentration (500 µg/ml) (Fig. 2C).

Based on the sequences of the linear peptides, two tetra-branched, multiple antigenic peptides, named MAP2-3 and MAP3, were synthesized using standard Fmoc chemistry. Each of the two MAPs contains four copies of a single peptide with C-terminus of each peptide linked with the non-immunogenic, lysine-based dendritic scaffold (Fig. 2D and Suppl. Fig. 1B). MAP2-3 peptide could be easily dissolved in PBS, deionized water or TBS buffer, whereas MAP3 peptide could only be dissolved in formic acid. Both MAP2-3 and MAP3 reacted with MA1-7401 mAb and LS-C102921 mAb at the same concentration (100 µg/ml) (Fig. 2F and Suppl. Fig. 1C). In contrast, both the lysine-based, tetra-branched scaffold for MAP (named MAPctrl. Fig. 2E) and MAP27 which mimics the conformation of PGN [21], failed to react with either of the two LTA-specific mAbs (Fig. 2F and Suppl. Fig. 1C).

3.3. Immunization with MAP2-3 raised IgG antibodies against LTA and S. aureus

Since MAP3 had poor aqueous solubility, in the rest of this study we focused on MAP2-3 and examined whether immunization with MAP2-3 can elicit the production of antigen-specific antibodies. As shown in Fig. 3A, pooled sera from MAP2-3-immunized mice reacted with MAP2-3 at a dilution of $1:10^3$ and reacted with *S. aureus* LTA at a dilution of $1:10^2$ (Fig. 3B). Sera from individual mouse also significantly bound to MAP2-3 or LTA (data not shown). In contrast, sera
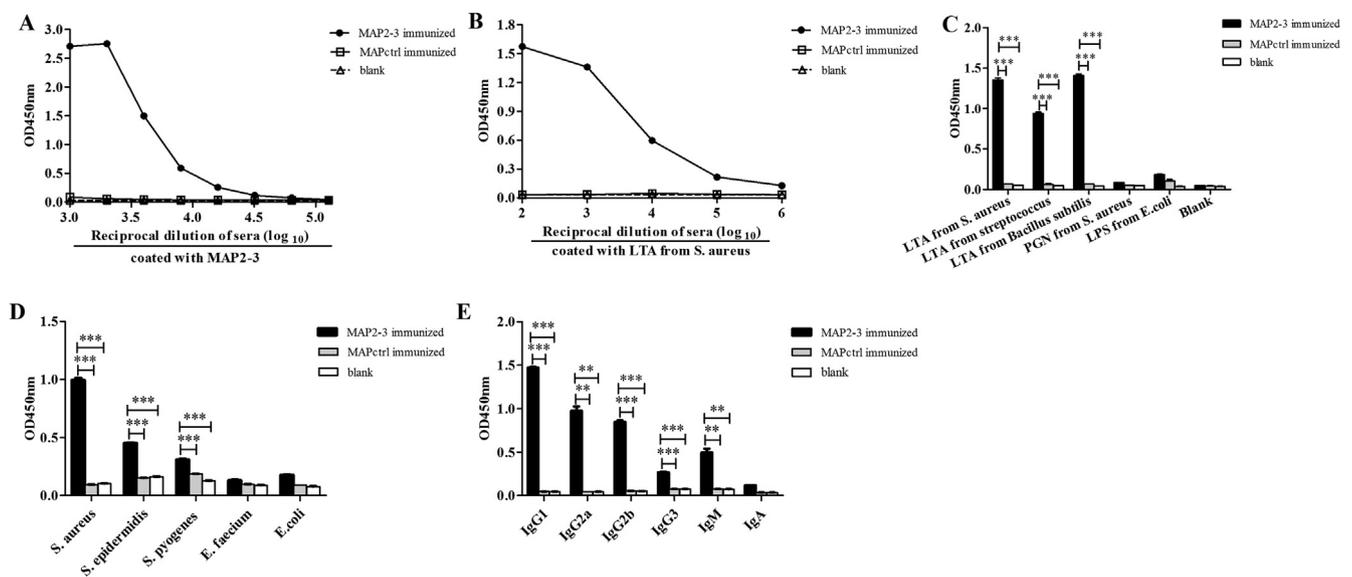

Fig. 3. Specificity of antisera detected by ELISA. Mice were immunized with MAP2-3, MAPctrl, or breed without immunization. Serum samples (n = 7–9 mice/group) were collected two weeks after the last immunization and pooled for ELISA. (A) Reactivity of serially diluted sera with MAP2-3. (B) Reactivity of serially diluted sera with LTA from *S. aureus*. (C) Reactivity of sera (1:100 dilution, n = 7–9 mice/group) with LTA from *S. aureus*, *Streptococcus*, or *B. subtilis*, PGN from *S. aureus*, or LPS from *E. coli*. (D) Binding of sera (1:100 dilution) to ultrasonic cleavage fragments of *S. aureus*, *S. epidermidis*, *S. pyogenes*, *E. faecium* or *E. coli*. (E) Immunoglobulin class of pooled anti-LTA sera (1:100 dilution) was determined by ELISA 15 days after the last immunization. For statistical analysis, one-way ANOVA followed by LSD was used for comparison between groups. *p*-values: **$p < 0.01$, ***$p < 0.001$.

from either the MAPctrl-immunized mice or the blank control mice failed to react with MAP2-3 or *S. aureus* LTA (Fig. 3A and 3B). As the basic structure of LTA is relatively conserved in many Gram positive bacteria, we next examined whether the serum samples could recognize LTA from different Gram positive bacteria. As shown in Fig. 3C, pooled anti-MAP2-3 sera significantly reacted with LTA from *S. aureus, Streptococcus*, and *B. subtilis* (Fig. 3C). Moreover, the reactivity of anti-MAP2-3 sera with LTA was specific since the sera did not react with other glycolipid components such as PGN from *S. aureus* and lipopolysaccharide (LPS) form *E. coli* (Fig. 3C). Anti-MAP2-3 sera also reacted with ultrasonic cleavage fragments of *S. aureus, S. epidermidis*, or *S. pyogenes* (Fig. 3D).

Ig class/subclass assay showed that MAP2-3 immunization elicited the production of LTA-specific IgG1, IgG2a, IgG2b, as well as low amount of IgG3 and IgM (Fig. 3E). However, IgA antibodies were not detected. The kinetics of IgG and IgM at different time points after immunization were also examined by ELISA. As shown in suppl. Fig. 2, IgG and IgM specific to MAP2-3 were continuously induced after the 3rd immunization (the 5th week of immunization).

The rabbit antisera specific to MAP2-3 were also prepared. As shown in Suppl. Fig. 3, the antisera significantly reacted with MAP2-3, LTA from *S. aureus,* or the ultrasonic product of *S. aureus*.

3.4. Vaccination with MAP2-3 protected mice against S. aureus systemic infection

Since LTA can synergize with PGN to induce sepsis [11], we next examined whether MAP2-3 immunization could confer protection in *S. aureus* systemic infection. In the septic shock model, about 60% of the MAP2-3-immunized BALB/c mice were still alive after 10 days of *S. aureus* lethal-challenge. In contrast, all of the MAPctrl-immunized mice and blank control mice died within three days of infection. MAP2-3-immunized mice had a significantly higher survival rate than the control (Fig. 4A, $p < 0.001$).

In the bacteremia model, MAP2-3 vaccination significantly reduced the bacterial load in kidney, spleen, and lungs (Fig. 4B-4D). Histopathological image analysis on kidney sections further demonstrated that MAP2-3-immunized mice had fewer numbers of abscess lesion and smaller sizes of abscess compared to the control mice (Fig. 4E).

We also examined the protective effect of active immunization in another mice strain C57BL/6. Similarly, MAP2-3 immunization significantly increased the survival rate (Suppl. Fig. 4A), decreased the bacterial load in tissues (Suppl. Fig. 4B–4D), and reduced the infiltration of inflammatory cells in kidney of mice infected with *S. aureus* (Suppl. Fig. 4E).
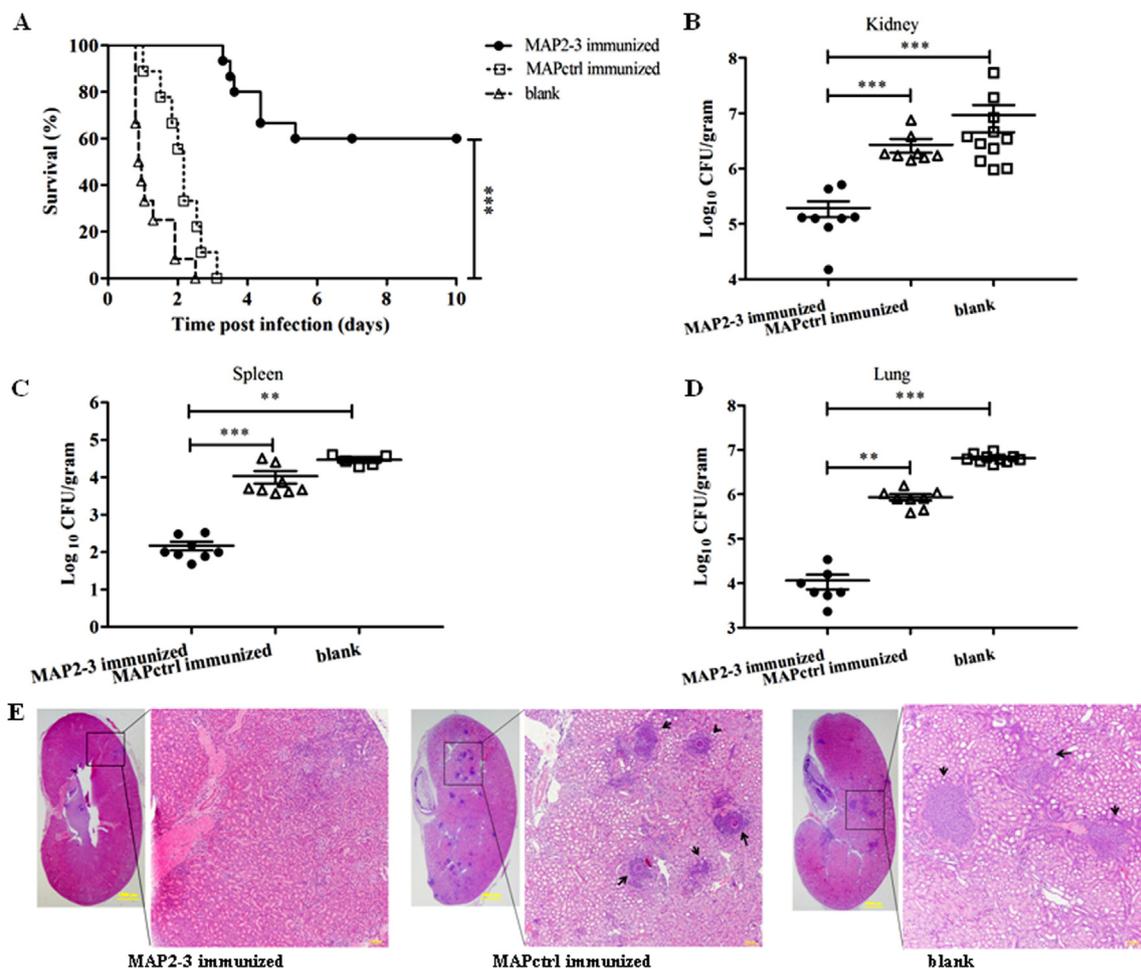

Fig. 4. Immunization with MAP2-3 protected BALB/c mice against *S. aureus* systemic infection. BALB/c mice were infected *S. aureus* via tail vein 7 days after the last immunization. The survival rate and the bacterial counts in organs were determined. (A) Survival analysis of mice (n = 10/group) with or without immunization after lethal-dose infection with *S. aureus* (1×10^8 CFU/mouse). (B-D) Bacterial counts in kidney (B), spleen (C) and lung (D) were measured three days after the infection of *S. aureus* (2×10^7 CFU/mouse, n = 5–11 mice/group). (E) Representative photos of hematoxylin-eosin stained kidney sections from mice four days after the infection of *S. aureus*. Magnification of images is $10\times$ for the entire kidney and $100\times$ for the zoomed-in photos. Infiltration of inflammatory cells in kidney and abscess are marked with arrows. Log-rank tests were conducted to compare the survival rates between different groups. Mann-Whitney U tests were performed to compare bacterial counts between two groups. Representative results from one of the three experiments are shown. *p*-values: $^{**}p < 0.01$, $^{***}p < 0.001$.

3.5. MAP2-3 immunization protected mice from infection by eliciting functional antibodies

Since MAP2-3 immunization could induce LTA-specific antibodies (Fig. 3B, 3C and Suppl. Fig. 3B), we next examined whether rabbit anti-MAP2-3 sera could enhance neutrophil cells to kill *S. aureus* by using *in vitro* opsonophagocytosis assay. As shown in Fig. 5A, the killing percentage of *S. aureus* mediated by rabbit anti-sera alone was significantly higher than that by normal rabbit sera (NRS) (Fig. 5A), while adding complement to the antisera did not markedly improve the killing rate (Fig. 5A, 35.4% ± 8.3 *vs.* 38.9% ± 9.4, *p*-value = 0.55). These results indicate that the protective effect of MAP2-3 immunization might be mediated partly through the Fc receptor-mediated phagocytosis. In addition to rabbit anti-MAP2-3, mouse anti-MAP2-3 sera could also improve the neutrophil cells to kill *S. aureus* (data not shown).

We next evaluated the protective effect of passive immunization by using three different infection models. In bacteremia model, the bacterial load in blood, lung and kidney from the mice that received anti-MAP2-3 sera was significantly lower than that from the mice that received normal rabbit sera (NRS) or PBS (Fig. 5B–5D). There was no significant difference in bacterial counts in spleens among the three groups (Fig. 5E).

Since *S. aureus* is commonly involved in respiratory infections, we also evaluated the protective effect of antisera in a pneumonia model. Bacterial load in the lungs of mice that received MAP2-3 antisera was significantly lower than that from the mice received NRS or PBS (Fig. 6A). Moreover, administration of anti-MAP2-3 sera significantly reduced acute lung injury and infiltration of inflammatory cells in lungs as shown in the gross histology pictures and H&E stained tissue sections (Fig. 6B and 6C).

We next examined the effect of passive immunization on skin infection [26]. As shown in Fig. 7A, passive immunization with anti-MAP2-3 sera significantly reduced the abscess volume and dermonecrosis area when compared to those received PBS or NRS. Moreover, passive immunization with anti-MAP2-3 sera also
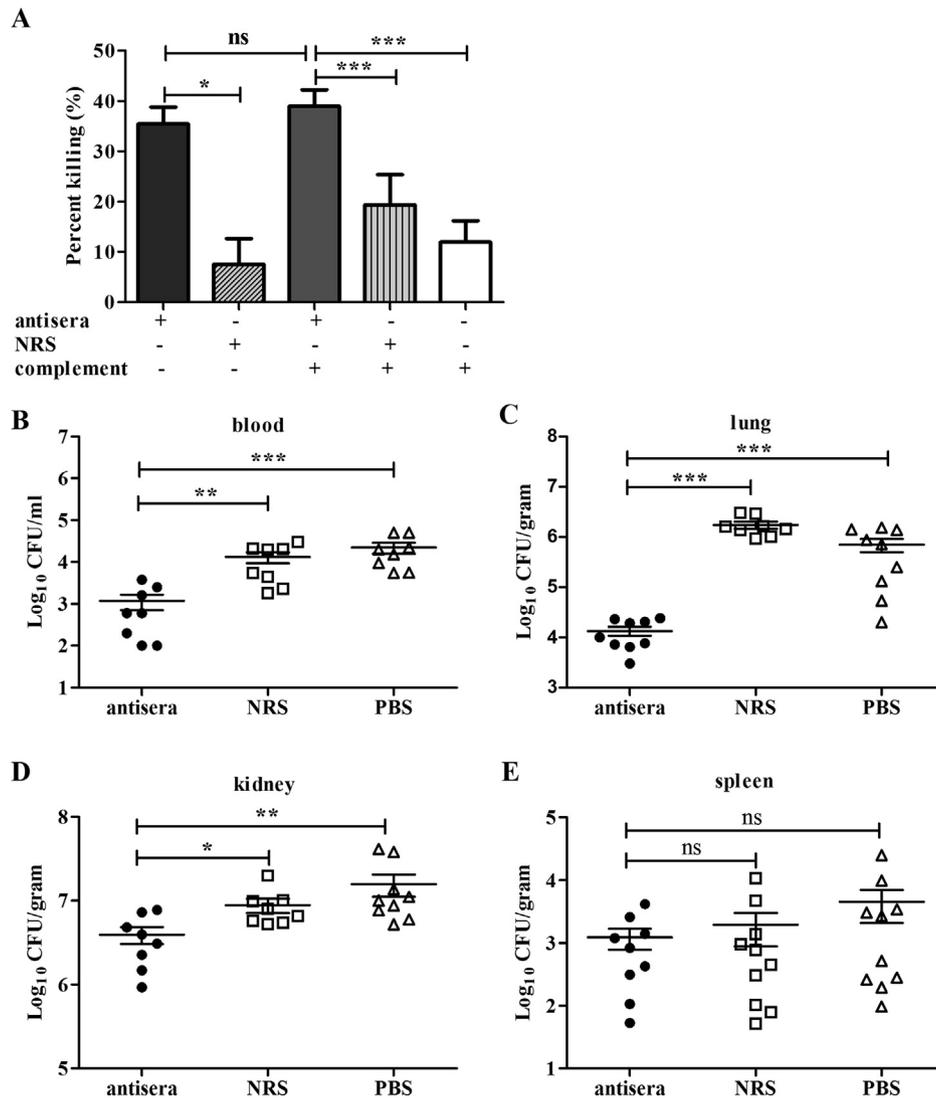

Fig. 5. Anti-MAP2-3 sera mediated opsonophagocytosis and conferred protection in *S. aureus* bacteremia model. (A) Bacteria were opsonized with rabbit anti-MAP2-3 sera (final dilution was 1:400) in the absence or presence of complement. In opsonophagocytosis assay, bacteria were first incubated with antisera at room temperature for 10 min. Purified mouse neutrophils used as effector cells (the effector to target cell ratio was 100:1) together with fresh guinea pig serum (final concentration: 1%) were then added and incubated for 1 h. The killing percentage was measured. The result was the average of two independent experiments. Data were analyzed using nonparametric tests for comparisons between two different groups. (B–E) Bacterial counts in blood and tissues in systemic infection model. Rabbit antisera (100 µl/mice) were given to the mice 24 h prior to *S. aureus* challenge via tail vein. The bacterial counts in blood (B) and lung (C) were measured 1 day after the infection. The bacterial counts in kidney (D) and spleen (E) were determined 3 days after the infection. Results are expressed with mean ± SEM. n = 8–10 mice/group. Data were analyzed using Mann-Whitney U for comparisons between two different groups. *p*-values: *p < 0.05, **p < 0.01, ***p < 0.001; ns, no significant difference. NRS represents normal rabbit serum.
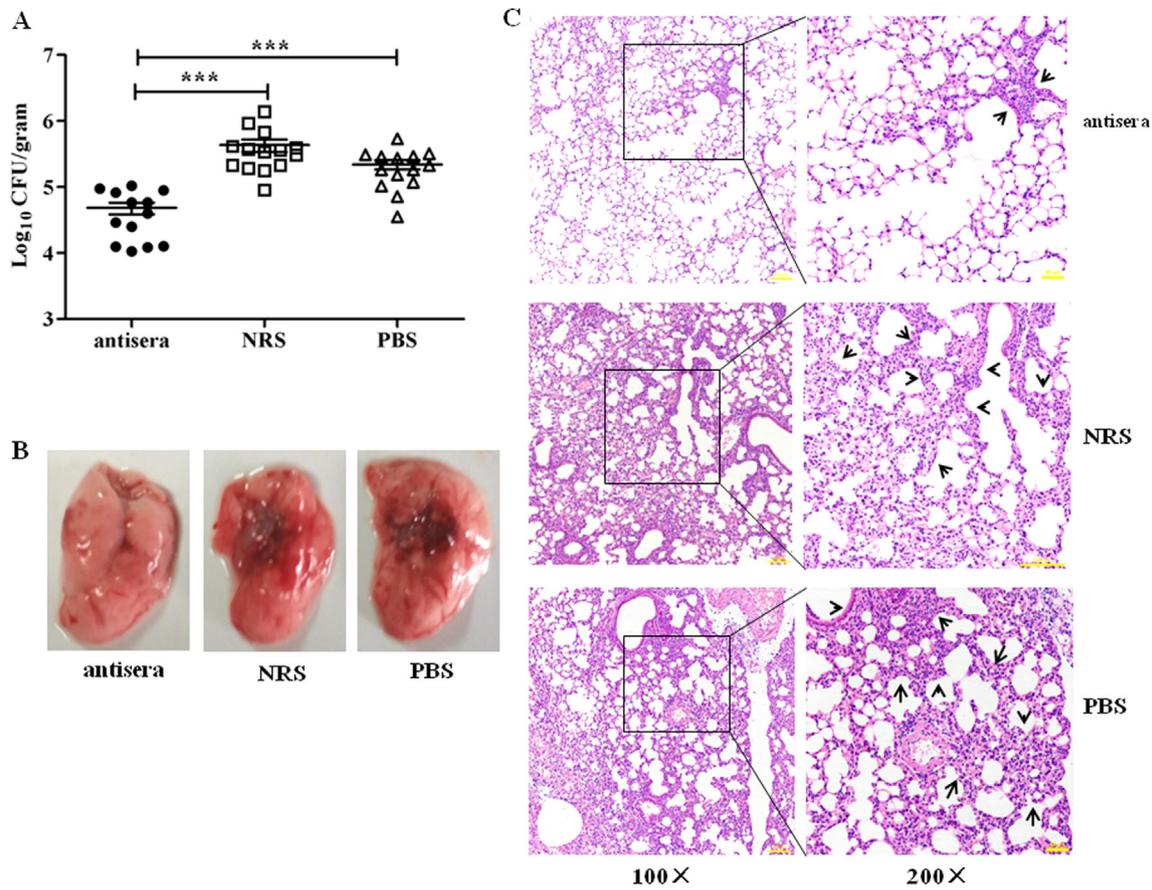
Fig. 6. Passive transfer with anti-MAP2-3 sera protected mice against infection in pneumonia model. Bacterial inoculums (4×10^7 CFU/mouse) were pre-mixed with rabbit antisera (10 μl/mouse) and subsequently delivered into the mice through nose (n = 13–14 mice/group). (A) The bacterial load in lungs was determined one day after the infection. (B) Representative photos of lung isolated one day after the infection. Pulmonary congestion was clearly observed in lungs of mice administered with NRS or PBS. (C) Representative photos of H&E stained tissue sections from lungs isolated one day after the infection. Infiltration of inflammatory cells in pulmonary interstitium and alveolar wall thickening are marked with arrows. Results are expressed as mean ± SEM. Data were analyzed using nonparametric tests for comparisons between two different groups. Image magnification is 100× in left panel and 200× in right panel. ***$p < 0.001$. NRS represents normal rabbit serum.

reduced the bacterial counts in skins 5 days after the infection (Fig. 7B). In addition, as shown in gross histology pictures and H&E stained tissue sections, abscess and dermonecrosis was substantially decreased in the mice received anti-MAP2-3 sera (Fig. 7C and 7D).

3.6. Immunization with MAP2-3 induced LTA-specific antibody-secreting cells (ASCs)

If MAP2-3 immunization can elicit specific IgG class antibodies, plasma cells specifically recognizing LTA or *S. aureus* would be developed and activated to produce antibodies after LTA or *S. aureus in vitro* re-stimulation. To test this hypothesis, ELISPOT assay was used to detect the specific antibody-secreting cells (ASCs). As shown in Fig. 8A and 8B, the number of anti-LTA ASCs from isolated splenocytes of the MAP2-3 immunized mice was significantly higher than that from the control mice (MAP2-3 *vs* MAPctrl, $30.36 \pm 6.07/10^6$ cells *vs* $1.33 \pm 0.79/10^6$ cells; MAP2-3 *vs* blank, $30.36 \pm 6.07/10^6$ cells *vs* $3.15 \pm 0.83/10^6$ cells, $p < 0.001$). Similarly, the number of anti-*S. aureus* ASCs from the MAP2-3 immunized mice was markedly higher than that from the control mice (Fig. 8C and 8D. MAP2-3 *vs* MAPctrl, $24.24 \pm 4.58/10^6$ cells *vs* $2.17 \pm 0.84/10^6$ cells; MAP2-3 *vs* blank, $24.24 \pm 4.58/10^6$ cells *vs* $1.92 \pm 0.57/10^6$ cells, $p < 0.001$). Taken together, immunization with MAP2-3 induced the specific humoral immune response.

4. Discussion

The notion that LTA can be used as a promising target for vaccination has been evaluated. For example, vaccination with LTA purified from *E. faecalis* elicited the production of opsonic antibodies [27]. To increase the immunogenicity, covalent conjugates of LTA with carrier proteins such as tetanus toxioid, BSA, and CRM197 have been created. Immunization with conjugate LTA induced specific antibodies, thereby conferring protections in different infection models [28–30]. Compared with glycan conjugation, our peptide mimic of LTA can be easily synthesized in large quantities with relatively lower toxicity and lower cost. Moreover, immunization with MAP2-3 significantly induced LTA-specific antibodies, and protected mice against *S. aureus* systemic infection. In addition, passive immunization with anti-MAP2-3 sera also protected BALB/c mice in three *S. aureus* infection models.

Since type I LTA structures are relatively conserved in most Gram-positive bacteria, it is possible that a single vaccine targeting LTA can be effective against multiple bacterial species. Theilacker's study shows that antibodies against *E. faecalis* LTA could bind to type I LTA from other Gram positive bacteria and opsonized *S. aureus*, *S. epidermidis*, and group B streptococci [27]. Consistently, we found that anti-MAP2-3 sera could react with LTA not only from *S. aureus*, but also from *Streptococcus* and *B. subtilis* (Fig. 3C). We also found that immunization of MAP2-3 reduced bacterial load in kidney from mice infected with *Enterococcus faecalis,* a bacterium that
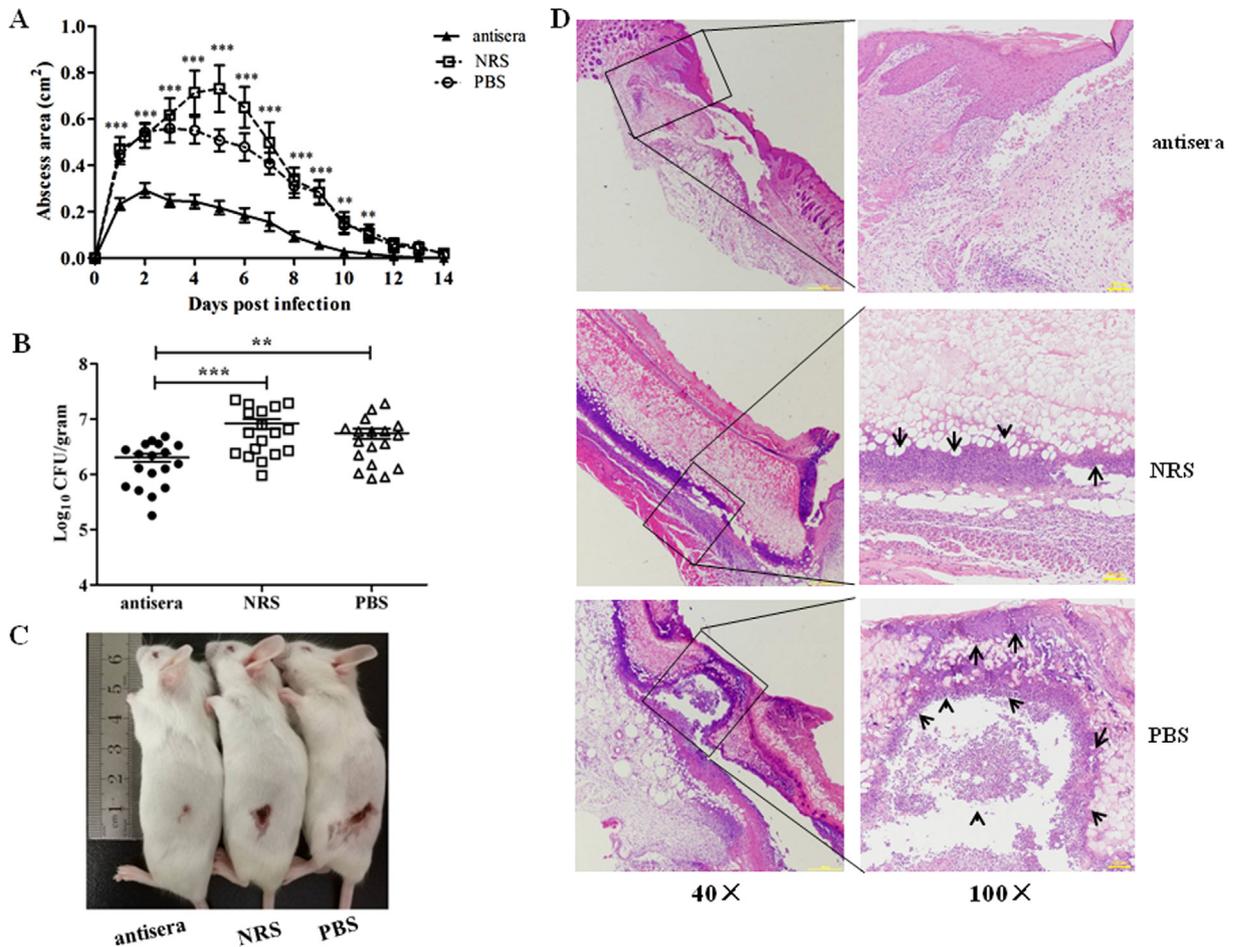
Fig. 7. Passive transfer with anti-MAP2-3 sera protected mice against infection in skin abscess model. Bacterial inoculums (2×10^7 CFU/mouse) were pre-mixed with rabbit antisera (50 µl/mouse) and subsequently administered to mice via subcutaneous injection. (A) Area of abscess and dermonecrosis was monitored once a day for two weeks (n = 10 mice/group). (B) The bacterial count in skin was measured five days after the infection (n = 18 mice/group). (C) Representative photos of mice five days after the infection. (D) Representative photos of H&E stained skin sections from mice five days after the infection. In mice administered with rabbit anti-MAP2-3 sera, discrete inflammatory cells under the skin were observed. In mice administered with NRS or PBS, tissue necrosis (NRS) and abscess foci (PBS) are marked with arrows. Results are expressed as mean ± SEM. Data were analyzed using nonparametric tests for comparisons between two different groups. Magnification of images in Fig. 7C is 40× in left panel and 100× in right panel. **$p < 0.01$, ***$p < 0.001$. NRS represents normal rabbit serum.

has a type I LTA structure (data not shown). These results suggest that immunization with MAP2-3 might be advantageous in developing a vaccine that would elicit antibodies that cross-react to a number of Gram-positive pathogens. However, immunization targeting a broad spectrum of bacteria might potentially impact on the microbiome composition of human host. Thus, the specificity and the safety of MAP2-3 immunization need to be elucidated in depth in the future.

We have detected LTA-specific IgG1, IgG2a, IgG2b, IgG3 and IgM in the sera after the immunization (Fig. 3E). ASCs specifically recognizing LTA or *S. aureus* were also developed and activated to produce specific IgG after *in vitro* re-stimulation (Fig. 8). These results suggest that active immunization with MAP2-3 resulted in the induction of a significant primary LTA-specific IgG response. An interesting question will be whether the immunization could also induce a cellular immune response. To test this hypothesis, we examined the cytokine production in splenocytes isolated from immunized mice after re-stimulation by LTA or *S. aureus*. We found that both LTA and *S. aureus* could significantly stimulate the production of IL-6 (data not shown). However, we failed to detect the production of IL-2 and IFN-γ, two key cytokines that secreted by T cells in response to infections. Ramanathan A et al's work

showed that vaccination with a MAP prophylactic antigen against lymphatic filariasis could elicit protective humoral response but low T cell proliferation. The low proliferation of T cells could be attributed to T/B epitope arrangement on the construct of MAP, MHC restriction, or incomplete signal delivery by T cell receptor [31]. Unlike Ramanathan A's work, MAP2-3 consists of only one sequence with four copies. Thus, whether MAP2-3 can be recognized by antigen presenting cells such as dendritic cells, and if so, whether MAP2-3 immunization induces cellular immune response, as well as how it induces a cellular immune response need to be further explored in the future studies.

5. Conclusion

In this work, a LTA-memitic, tetra-branched antigenic peptide (MAP2-3), derived from a phage peptide library screen, was synthesized. Immunization with MAP2-3 elicited specific antisera against LTA and protected mice from *S. aureus* systemic infection. Passive immunization with anti-MAP2-3 sera also protected mice in three *S. aureus* infection models. This work will provide a new option for designing multi-antigen vaccines against *S. aureus*.
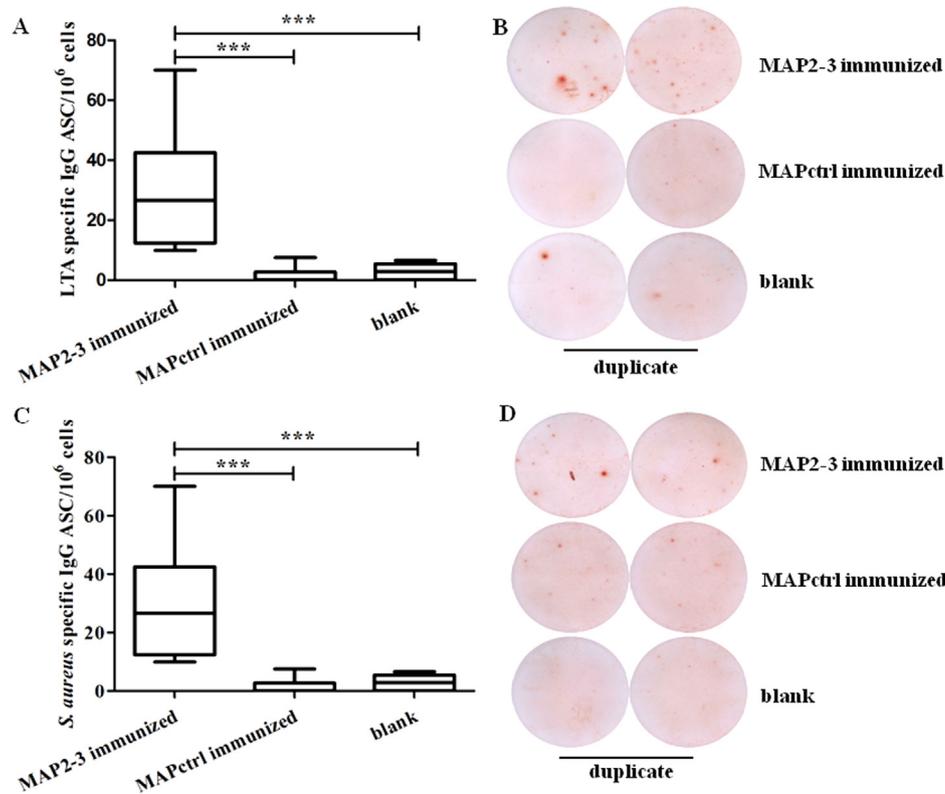
Fig. 8. Frequency of LTA-specific and *S. aureus*-specific IgG antibody-secreting cells (ASCs). BALB/c mice were immunized with MAP2-3 or MAPctrl for five times with 2-week intervals. After one month of the last immunization, the splenocytes were isolated. 4×10^5/well cells were added to ELISPOT plates precoated with LTA or heat-killed *S. aureus* and incubated at 37 °C for 24 h. Antibody-secreting cells (ASCs) were detected with HRP-conjugated goat anti-mouse IgG. (A) Average frequency of LTA-specific IgG secreting cells in spleens (n = 3–5 mice/group). (B) Representative photos of LTA-specific IgG ELISPOT results. (C) Average frequency of *S. aureus*-specific IgG secreting cells in spleens (n = 3–5 mice/group). (D) Representative photos of *S. aureus*-specific IgG ELISPOT results. Data are expressed as mean ± SEM. Nonparametric test was used for comparisons between different groups. ***$p < 0.001$.

Declaration of Competing Interest

The peptide sequences (VHWDFRQWWQPS and GHWDFRQWWQPS) have been submitted for application for the national invention patent (International Application No. CN106632623A). Sequence of GHKEDRQWCQHS has been submitted for application for the national invention patent (International Application No. CN106478788A).

Author contributions

Conceived and designed the experiments: BY Liu. Performed the experiments: XY Yi, ZX Huang, XR Hou, P Zhu, XY Wang and HB Luo. Analyzed the data: XY Yi and ZX Huang. Contributed reagents/materials/analysis tools: P Zhu. Wrote the paper: BY Liu.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.06.024.

References

[1] Lowy FD. *Staphylococcus aureus* infections. N Engl J Med 1998;339(8):520–32.
[2] Foster TJ. Colonization and infection of the human host by staphylococci: adhesion, survival and immune evasion. Vet Dermatol 2009;20(5–6):456–70.
[3] DeLeo FR, Otto M, Kreiswirth BN, Chambers HF. Community-associated meticillin-resistant *Staphylococcus aureus*. Lancet 2010;375(9725):1557–68.
[4] Su CH, Chang SC, Yan JJ, Tseng SH, Chien LJ, Fang CT. Excess mortality and long-term disability from healthcare-associated *Staphylococcus aureus* infections: a population-based matched cohort study. PLoS One 2013;8(8):e71055.
[5] Percy MG, Grundling A. Lipoteichoic acid synthesis and function in gram-positive bacteria. Annu Rev Microbiol 2014;68:81–100.
[6] Schneewind O, Missiakas D. Lipoteichoic acids, phosphate-containing polymers in the envelope of gram-positive bacteria. J Bacteriol 2014;196(6):1133–42.
[7] Lebeer S, Verhoeven TL, Perea VM, Vanderleyden J, De Keersmaecker SC. Impact of environmental and genetic factors on biofilm formation by the probiotic strain Lactobacillus rhamnosus GG. Appl Environ Microbiol 2007;73(21):6768–75.
[8] Abachin E, Poyart C, Pellegrini E, Milohanic E, Fiedler F, Berche P, et al. Formation of D-alanyl-lipoteichoic acid is required for adhesion and virulence of Listeria monocytogenes. Mol Microbiol 2002;43(1):1–14.

[9] Fabretti F, Theilacker C, Baldassarri L, Kaczynski Z, Kropec A, Holst O, et al. Alanine esters of enterococcal lipoteichoic acid play a role in biofilm formation and resistance to antimicrobial peptides. Infect Immun 2006;74(7):4164–71.

[10] Sheen TR, Ebrahimi CM, Hiemstra IH, Barlow SB, Peschel A, Doran KS. Penetration of the blood-brain barrier by *Staphylococcus aureus*: contribution of membrane-anchored lipoteichoic acid. J Mol Med (Berl) 2010;88(6):633–9.

[11] Thiemermann C. Interactions between lipoteichoic acid and peptidoglycan from *Staphylococcus aureus*: a structural and functional analysis. Microbes Infect 2002;4(9):927–35.

[12] Fukuda MN. Peptide-displaying phage technology in glycobiology. Glycobiology 2012;22(3):318–25.

[13] Hou Y, Gu XX. Development of peptide mimotopes of lipooligosaccharide from nontypeable Haemophilus influenzae as vaccine candidates. J Immunol 2003;170(8):4373–9.

[14] Menendez T, Santiago-Vispo NF, Cruz-Leal Y, Coizeau E, Garay H, Reyes O, et al. Identification and characterization of phage-displayed peptide mimetics of Neisseria meningitidis serogroup B capsular polysaccharide. Int J Med Microbiol 2011;301(1):16–25.

[15] Umair S, Deng Q, Roberts JM, Shaw RJ, Sutherland IA, Pernthaner A. Identification of peptide mimics of a glycan epitope on the surface of parasitic nematode larvae. PLoS One 2016;11(8):e162016.

[16] Yu RK, Usuki S, Itokazu Y, Wu HC. Novel GM1 ganglioside-like peptide mimics prevent the association of cholera toxin to human intestinal epithelial cells in vitro. Glycobiology 2016;26(1):63–73.

[17] Di John D, Wasserman SS, Torres JR, Cortesia MJ, Murillo J, Losonsky GA, et al. Effect of priming with carrier on response to conjugate vaccine. Lancet 1989;2(8677):1415–8.

[18] Fujita Y, Taguchi H. Current status of multiple antigen-presenting peptide vaccine systems: application of organic and inorganic nanoparticles. Chem Cent J 2011;5(1):48.

[19] Mahajan B, Berzofsky JA, Boykins RA, Majam V, Zheng H, Chattopadhyay R, et al. Multiple antigen peptide vaccines against Plasmodium falciparum malaria. Infect Immun 2010;78(11):4613–24.

[20] Oscherwitz J, Yu F, Cease KB. A synthetic peptide vaccine directed against the 2ss2-2ss3 loop of domain 2 of protective antigen protects rabbits from inhalation anthrax. J Immunol 2010;185(6):3661–8.

[21] Wang XY, Huang ZX, Chen YG, Lu X, Zhu P, Wen K, et al. A Multiple antigenic peptide mimicking peptidoglycan induced T cell responses to protect mice from systemic infection with *Staphylococcus aureus*. PLoS One 2015;10(8): e136888.

[22] Bagnoli F, Fontana MR, Soldaini E, Mishra RP, Fiaschi L, Cartocci E, et al. Vaccine composition formulated with a novel TLR7-dependent adjuvant induces high and broad protection against *Staphylococcus aureus*. Proc Natl Acad Sci USA 2015;112(12):3680–5.

[23] Mishra RP, Mariotti P, Fiaschi L, Nosari S, Maccari S, Liberatori S, et al. *Staphylococcus aureus* FhuD2 is involved in the early phase of staphylococcal dissemination and generates protective immunity in mice. J Infect Dis 2012;206(7):1041–9.

[24] Zhang F, Jun M, Ledue O, Herd M, Malley R, Lu YJ. Antibody-mediated protection against *Staphylococcus aureus* dermonecrosis and sepsis by a whole cell vaccine. Vaccine 2017;35(31):3834–43.

[25] Huang Z, Yi X, Chen Y, Hou X, Wang X, Zhu P, et al. Pretreatment of Pam3CSK4 attenuates inflammatory responses caused by systemic infection of methicillin-resistant *Staphylococcus aureus* in mice. Biomed Pharmacother 2017;95:1684–92.

[26] Acree ME, Morgan E, David MZ. *S. aureus* infections in Chicago, 2006–2014: increase in CA MSSA and decrease in MRSA incidence. Infect Control Hosp Epidemiol 2017;38(10):1226–34.

[27] Theilacker C, Kropec A, Hammer F, Sava I, Wobser D, Sakinc T, et al. Protection against *Staphylococcus aureus* by antibody to the polyglycerolphosphate backbone of heterologous lipoteichoic acid. J Infect Dis 2012;205(7):1076–85.

[28] Chen Q, Dintaman J, Lees A, Sen G, Schwartz D, Shirtliff ME, et al. Novel synthetic (poly)glycerolphosphate-based antistaphylococcal conjugate vaccine. Infect Immun 2013;81(7):2554–61.

[29] Laverde D, Wobser D, Romero-Saavedra F, Hogendorf W, van der Marel G, Berthold M, et al. Synthetic teichoic acid conjugate vaccine against nosocomial Gram-positive bacteria. PLoS One 2014;9(10):e110953.

[30] Broecker F, Martin CE, Wegner E, Mattner J, Baek JY, Pereira CL, et al. Synthetic lipoteichoic acid glycans are potential vaccine candidates to protect from clostridium difficile infections. Cell Chem. Biol. 2016.

[31] Ramanathan A, Immanuel C, Rao DN, Kaliraj P. Dissecting the immune response elicited by WbALT-2, ALT MAP in clinical populations and mouse model: a prophylactic measure against lymphatic filariasis. Lymphat Res Biol 2015;13(2):120–5.